Both Erk and p38 Kinases Are Necessary for Cytokine Gene Transcription

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A critical feature of sepsis-induced acute lung injury is the release of cytokines from endotoxin (LPS)-stimulated alveolar macrophages (AM). LPS is also known to activate various members of the mitogen-activated protein kinase (MAPK) family in other types of cells. In this study, we evaluated whether multiple members of the MAPK family regulate cytokine gene expression in LPS-stimulated AM. We found that LPS activates both the extracellular signal–regulated kinase (Erk) and p38 kinases, and that this activation is augmented when the cells are cultured in serum. Inhibition of either the Erk (with PD98059) or p38 (with SB203580) kinase pathway resulted in only a partial reduction in cytokine (interleukin-6 and tumor necrosis factor) messenger RNA accumulation and cytokine release, whereas inhibition of both pathways simultaneously resulted in a decrease in cytokine gene expression to near-control levels. Nuclear run-on assays showed that the effect of these MAPK pathways on LPS-induced expression of the cytokine genes was attributable, at least in part, to regulation of gene transcription. These findings suggest that activation of both the Erk and p38 kinase pathways is necessary for optimal cytokine gene expression in LPS-stimulated human AM, and that the MAPK pathways play a critical role in the inflammatory response that occurs in sepsis-induced acute lung injury. **Carter, A. B., M. M. Monick, and G. W. Hunninghake. 1999. Both ERK and p38 kinases are necessary for cytokine gene transcription. Am. J. Respir. Cell Mol. Biol. 20:751–758.**

One form of lung injury that often results from sepsis is the adult respiratory distress syndrome (ARDS) (1). Although many factors contribute to the inflammatory process that occurs in ARDS, one of the early and ongoing factors is the release of cytokines from alveolar macrophages (AM). Endotoxin (LPS), which is released during sepsis, is a major stimulus for the release of cytokines from AM, and a prolonged release of cytokines by AM has been associated with a more adverse outcome in patients with this disease (2–4).

One closely related group of kinases known to be activated by LPS in other types of cells are the mitogen-activated protein kinases (MAPKs) (5–15). Three important groups of kinases compose the MAPK family, including the p42/44 (also known as extracellular signal–regulated kinase [Erk]) kinases, the c-Jun N-terminal kinases (JNK), and the p38 kinases. Prior studies in monocytes (5, 6) have shown that the p38 MAPK pathway is critical for LPS-induced cytokine release. In these studies, specific inhibition of the p38 kinase pathway by SB203580 resulted in reduced cytokine release secondary to a defect in translation (5). No other studies, using any type of cells, have evaluated whether other MAPK pathways are required for optimal LPS-induced expression of cytokine genes. The only potentially relevant study is by Reimann and colleagues (15), who used BAC-1.2F5 macrophages and showed that expression of *v-raf* resulted in activation of the Erk kinases and the release of small amounts of interleukin (IL)-1β and tumor necrosis factor (TNF)-α. Exposure of these *v-raf*–infected cells to LPS resulted in expression of greater amounts of cytokine messenger RNAs (mRNAs). BAC-1.2F5 macrophages infected with *v-raf*, however, exhibit a suppression of LPS-induced Erk kinase activation (15). Thus, it is not clear from this study whether the effect of LPS was mediated through the Erk kinase pathway or some other pathway.

Other studies have linked immunoglobulin (Ig) G–mediated or IgE-mediated activation of Erk kinases in leukocytes and murine macrophages or mast cells, respectively, to the expression of the TNF gene (16–18). These effects

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Abbreviations: alveolar macrophages, AM; activator protein-1, AP-1; adult respiratory distress syndrome, ARDS; adenosine triphosphate, ATP; extracellular signal–regulated kinase, Erk; fetal calf serum, FCS; immunoglobulin, Ig; interleukin, IL; c-Jun N-terminal kinases, JNK; endotoxin, LPS; mitogen-activated protein kinase, MAPK; MAPK kinase, MEK; messenger RNA, mRNA; nuclear factor, NF; sodium dodecyl sulfate–polyacrylamide gel electrophoresis, SDS–PAGE; saline sodium citrate, SSC; saline sodium phosphate ethylenediaminetetraacetic acid, SSPE; tumor necrosis factor, TNF.

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of IgG or IgE on TNF gene expression could be inhibited by PD98059, a selective inhibitor of the MEK→Erk kinase pathway. In the studies that used IgG as a stimulus, activation of the p38 kinase pathway was not linked to expression of the TNF gene (16, 17); whereas in the study that used IgE as a stimulus, the p38 kinase negatively regulated the Erk kinases and TNF gene expression (18). To our knowledge, no studies have linked expression of the Erk kinase pathway to cytokine gene expression in cells stimulated with LPS. Furthermore, no studies have shown that both the Erk and p38 kinase pathways contribute to optimal cytokine gene expression with any type of stimulus.

We hypothesized that activation of both the Erk and p38 kinase pathways may be necessary for optimal LPS-induced cytokine gene expression in AM. For these studies we used human AM because they are relevant cells that release cytokines in the lungs of patients with sepsis-induced acute lung injury. We found that LPS activates both Erk and p38 kinases in AM, and that this activation is enhanced by the presence of serum. LPS-induced Erk kinase activity could be reduced to control levels with PD-98059, a specific inhibitor of the MEK→Erk kinase pathway, and p38 kinase activity could be reduced to control levels with SB203580, a specific inhibitor of the p38 kinase pathway. LPS increased amounts of IL-6 and TNF mRNAs and proteins in AM. Although inhibition of either the Erk or p38 kinases resulted in a small reduction, inhibition of both pathways reduced LPS-induced cytokine gene expression to near-control levels. The effects of PD98059 and SB203580 on cytokine gene expression were due, at least in part, to inhibition of gene transcription.

Materials and Methods

Isolation of AM

The use of normal volunteers to obtain AM by bronchoalveolar lavage (BAL) and human blood monocytes by phlebotomy was approved by the Human Subjects Review Board of the University of Iowa College of Medicine (Iowa City, IA). AM and monocytes were obtained from normal volunteers who met the following criteria: (*1*) age between 18 and 45 yr; (*2*) no history of cardiopulmonary disease or other chronic disease; (*3*) no prescription or nonprescription medication except oral contraceptives; (*4*) no recent or current evidence of infection; and (*5*) lifetime nonsmoker. The volunteers underwent phlebotomy and/or fiberoptic bronchoscopy with BAL in subsegments of the right upper lobe, right middle lobe, and lingula after receiving intramuscular atropine, 0.6 mg, and local anesthesia. Each subsegment of the lung was lavaged with five 20-ml aliquots of normal saline, and the first aliquot in each was discarded. The percentage of AM was determined by Wright–Giemsa stain and varied from 90 to 98%.

Expression of MAPK

AM were cultured at 37°C for 15 to 30 min in Roswell Park Memorial Institute (RPMI)-1640 medium with 5% fetal calf serum (FCS), and they were stimulated with *Escherichia coli* serotype 026:B6 LPS (Sigma, St. Louis, MO) at a dose of 1 μg/ml. The MEK inhibitor PD98059 (New England Biolabs, Beverly, MA), at 10 μM, and the p38 inhibitor SB203580 (Calbiochem, La Jolla, CA), at 1 μM, were added 1 h before stimulation with LPS. These concentrations of PD98059 and SB203580 are approximately twice the concentration that is necessary to inhibit their respective kinases (IC_{50}), according to the product information and relevant literature (6, 18–23). In our AM and monocytes, these concentrations represented approximately twice the IC_{50} as well. These concentrations of inhibitors were not toxic to the cells, as evaluated by trypan blue stain. Experiments with short time courses, such as kinase assays, had cell viabilities greater than 90% in all groups. Experiments with long time courses, such as enzyme-linked immunosorbent assays (ELISAs), had cell viabilities ranging from 60 to 80% in all groups. The cells were harvested, resuspended in a lysis buffer (1% NP-40, 0.15 M NaCl, 0.05 M Tris [pH 7.4], 100 μg/ml phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 0.4 M Na_3VO_4, 10 mM NaF, and 10 mM sodium pyrophosphate), sonicated, and placed on ice for 20 min. After centrifuging at 14,000 rpm at 4°C for 10 min to remove cellular debris, the lysates were stored at −70°C. Erk2 and p38 were immunoprecipitated from the lysates (600 μg) overnight at 4°C with their respective antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) bound to Gammabind with sepharose (Pharmacia Biotech, Uppsala, Sweden). The sepharose pellet was placed in a kinase buffer (20 mM $MgCl_2$, 25 mM *N*-2-hydroxyethylpiperazine-*N′*-ethane sulfonic acid, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenylphosphate, 0.1 mM $NaVO_4$, and 2 mM dithiothreitol) with [γ-^{32}P]adenosine triphosphate (ATP) (5 μCi/sample; New England Nuclear/DuPont, Boston, MA), ATP, and myelin basic protein (MBP; Sigma). Kinase activity was assayed by phosphorylation of MBP for both Erk2 and p38. After 15 min, the kinase reaction was stopped with the addition of ethylenediaminetetraacetic acid (EDTA)-containing Laemmli sample buffer, and the samples were heated to 95°C for 5 min to separate the protein from the sepharose. The samples were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) discontinuous gel at 45 mA. The gels were subsequently vacuum-dried and exposed to autoradiographic film at room temperature. Western blot analysis was performed simultaneously, with equal aliquots taken from each sample after immunoprecipitation to ensure equal loading of the kinases. The samples for the kinase assay and the Western analysis were run on separate SDS–PAGE discontinuous gels.

Expression of Cytokine mRNA

Whole-cell RNA was isolated using RNA Stat-60 according to the manufacturer's instructions (Tel-test "B"; Friendswood, TX). Cells were lysed in RNA Stat-60 solution, containing phenol and guanidinium thiocyanate. The mixture was then shaken vigorously, allowed to sit for 2 to 3 min, and then centrifuged at 12,000 rpm for 15 min at 4°C. The RNA was removed from the upper aqueous layer, precipitated with isopropanol for 30 min, centrifuged at 12,000 rpm for 10 min at 4°C, and washed with 75% ethanol. The isolated RNA was fractionated in a 1.5% denaturing agarose gel containing 2.2 M formaldehyde. An RNA ladder (0.24 to 9.5 kb; Life Technologies,

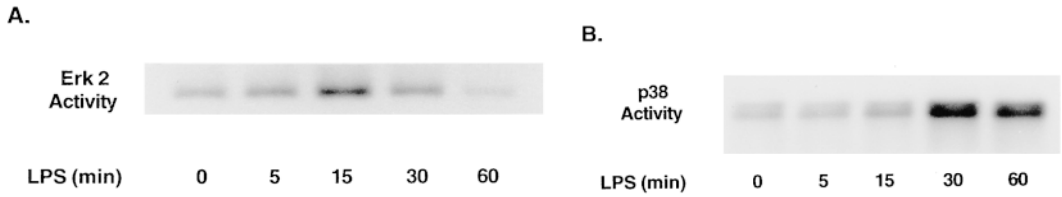

Figure 1. Activation of MAPK by LPS in human AM. (*A*) Erk2 kinase is activated by LPS and has maximal activity at 15 min. (*B*) p38 kinase is activated by LPS and has maximal activity at 30 min, and it remains active up to 60 min. These figures are representative of four different experiments.

Gaithersburg, MD) was included as a molecular-size standard. RNA loading was confirmed by equivalent ethidium bromide staining in each lane. The RNAs were transferred to GeneScreen Plus paper (New England Nuclear/DuPont) as suggested by the manufacturer. IL-6 and TNF complementary DNA (cDNA) probes (generated by polymerase chain reaction [PCR]; Clontech, Palo Alto, CA) were labeled with [γ-^{32}P]cytidine triphosphate (CTP) (New England Nuclear/DuPont) by random primer method. Blots were prehybridized for 3 h at 42°C (10 ml formamide, 5 mM NaCl, 4 ml 50% dextran, 10% SDS, 1 M Tris [pH 7.0], and 0.4 ml 50× Denhardt's solution) and then hybridized with the labeled probe overnight at 42°C. The filters were washed twice with 1× saline sodium citrate (SSC) at 25°C, twice with 1× SSC plus 1% SDS at 65°C, and then once with 0.1× SSC at 25°C. The filters were exposed to autoradiographic film at −70°C.

Nuclear Run-On Assay

For these studies, AM were cultured in RPMI medium with 5% FCS for 3 h at 37°C in the presence or absence of LPS and in the presence or absence of PD98059 or SB-203580. The cells were harvested and washed with ice-cold phosphate-buffered saline. The cells were lysed in a lysis buffer (10 mM Tris [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40), and left on ice for 5 min. After centrifuging at 1,500 rpm for 5 min at 4°C, the nuclear pellet was resuspended in a glycerol storage buffer and stored at −70°C. To assay nuclear transcription, the nuclear pellets were thawed and placed in a reaction buffer (10 mM Tris [pH 8.0], 5 mM $MgCl_2$, 0.3 M KCl) with 1 mM of nucleotides (ATP, CTP, and guanosine triphosphate), and 10 μl of [γ-^{32}P]uridine triphosphate (400 μCi/sample; New England Nuclear/DuPont), and each sample was placed in an orbital shaker for 30 min at 30°C. The reaction was stopped with the addition of 1 mg/ml of ribonuclease (RNase)-free DNase I (Promega, Madison, WI) and 20 mg/ml of proteinase K (Life Technologies). RNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The RNA solution was then placed in 10% trichloroacetate/60 mM sodium pyrophosphate with 10 μl of 10 mg/ml transfer RNA and left on ice for 30 min. The precipitate was filtered onto 0.45-μm filters, and the ^{32}P-labeled RNA was eluted. The RNA was precipitated for 30 min on dry ice and centrifuged for 30 min at 9,000 rpm at 4°C. The pellet was resuspended in 1 ml of TES solution (10 mM N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid [pH 7.4], 10 mM EDTA, and 0.2% SDS). The samples were counted and equalized by counts per minute of ^{32}P-labeled RNA/ml. The equalized samples were then hybridized at 65°C for 36 h to IL-6 cDNA (generated by PCR; Clontech) that was previously bound to nitrocellulose. The nitrocellulose filters were washed six times in 1× saline sodium phosphate EDTA (SSPE) with 1% SDS at 65°C, once in 0.1× SSPE with 1% SDS at 65°C, once in 2× SSPE with RNase A 1.25 μg/ml at 37°C, and three times in 2× SSPE at room temperature. The filters were allowed to dry at room temperature and subsequently exposed to autoradiographic film at −70°C.

Expression of Cytokines

For these studies, AM were cultured in RPMI medium with 5% FCS for 24 h in the presence or absence of LPS and with and without inhibitors, as described above. The amounts of IL-6 and TNF in the supernatant of the cells were measured by ELISA (R&D Systems, Minneapolis, MN).

Statistical Analysis

All of the cytokine measurements are shown as means with standard error. Statistical comparisons were performed using an unpaired, one tailed *t* test with a probability value of $P < 0.05$ considered to be significant.

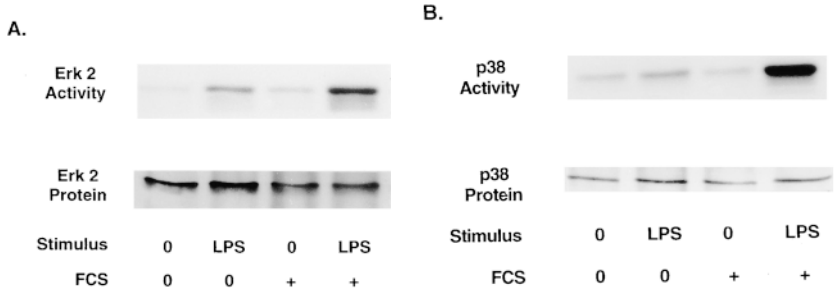

Figure 2. MAPK activity in human AM cultured in serum. (*A*) LPS alone can activate Erk2 kinase, but this activity is augmented in cells cultured in serum. (*B*) LPS alone can activate p38 kinase, but this activity is augmented in cells cultured in serum. Western blot analysis shows equal loading of proteins. These figures are representative of four different experiments.

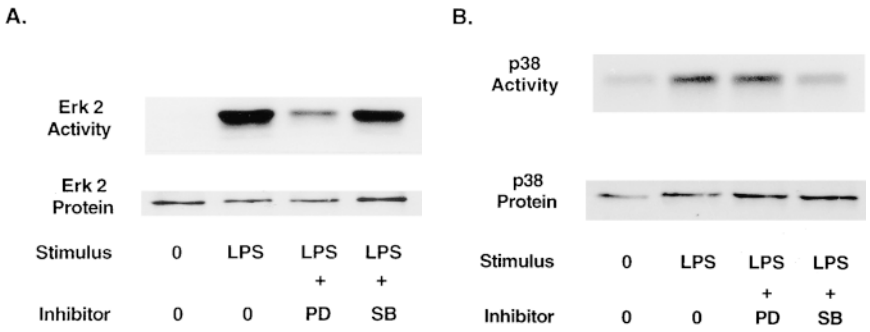

Figure 3. Selective inhibition of MAPK in human AM. (*A*) Erk2 activity is significantly reduced by PD98059 (PD), a specific inhibitor of the MEK→Erk pathway. There is no detectable effect on the p38 kinase activity. (*B*) p38 activity is significantly reduced by SB203580 (SB), a competitive inhibitor of p38 kinase. There is no detectable effect on the Erk2 activity. Western blot analysis shows equal loading of proteins. These figures are representative of four different experiments.

Results

MAPK Activation by LPS in Human AM

To determine the time at which maximal kinase activity is expressed, we stimulated AM with LPS for 5, 15, 30, and 60 min before evaluating kinase activity. For the Erk2 kinase, the greatest activity was at 15 min, but there was detectable kinase activity as early as 5 min and as late as 60 min after LPS stimulation (Figure 1A). For the p38 kinase, activity was maximal at 30 min and the kinase remained active at 60 min (Figure 1B). AM cultured in serum-free conditions with LPS expressed increased activity of both Erk2 and p38 kinases, but cells cultured in RPMI medium with 5% serum and LPS had much greater activation of these kinases (Figure 2). These are the first studies to show that LPS activates both Erk and p38 kinases in human AM. The studies further show that serum augments this effect of LPS.

Selective Inhibition of Erk and p38 Kinases in Human AM

To evaluate the role of the Erk and p38 kinase pathways in regulating the release of cytokines from LPS-stimulated AM, it was necessary to inhibit each pathway selectively. To determine whether the inhibitors were specific for each pathway, we performed kinase assays and Western blot analysis of both the Erk2 and p38 kinases. The LPS-induced Erk2 kinase activity could be significantly reduced by PD-98059, a specific inhibitor of the Erk kinase pathway (Figure 3A), and the p38 kinase activity could be significantly reduced by SB203580, a specific inhibitor of this pathway (Figure 3B). PD98059 had no detectable effect on activation of the p38 kinase, and SB203580 had no detectable effect on activation of the Erk kinase. These studies show that the inhibitors are effective and specific, and the results are consistent with prior studies that also showed the specificity of these inhibitors (5, 17, 18, 20, 24, 25).

Both Erk and p38 Kinase Pathways Are Necessary for Maximal LPS-Induced Cytokine Release in Human AM

To determine whether both the Erk and p38 kinase pathways regulate cytokine release, we measured the release of IL-6 and TNF from LPS-stimulated AM. As expected, LPS markedly increased the release of both cytokines. In-

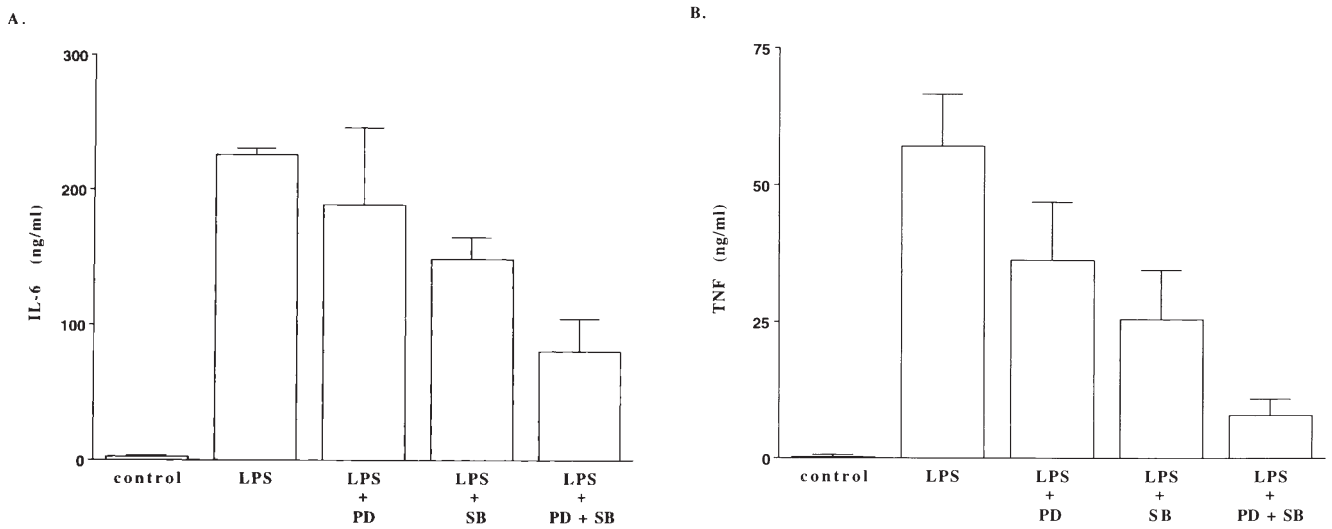

Figure 4. Effect of MAPK inhibition on LPS-induced cytokine release in human AM. (*A*) LPS increases the release of IL-6, and there is partial reduction with inhibition of either the Erk or the p38 kinase pathway. Inhibition of both pathways reduces IL-6 release to near-control levels ($P < 0.005$). (*B*) LPS increases the release of TNF, and there is partial reduction with inhibition of either the Erk or p38 kinase pathway. Inhibition of both pathways reduces TNF release to near-control levels ($P < 0.004$).

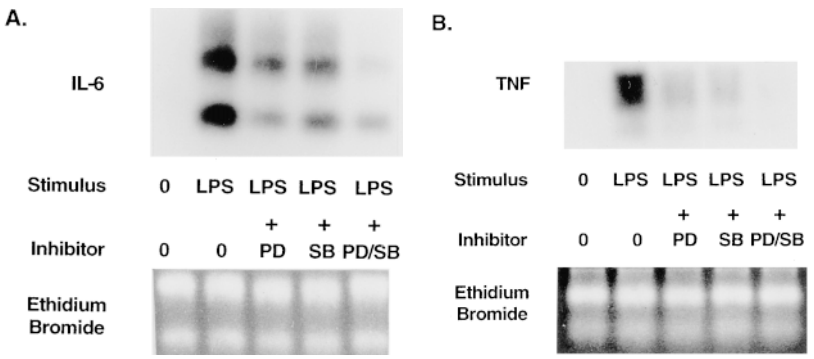

Figure 5. Effect of MAPK inhibition on cytokine mRNA accumulation in human AM. (*A*) LPS increases the accumulation of IL-6 mRNA, and there is partial reduction with inhibition of either the Erk or the p38 kinase pathway. Inhibition of both pathways reduces IL-6 mRNA to control levels. (*B*) LPS increases the accumulation of TNF mRNA, and there is a partial reduction with inhibition of either the Erk or the p38 kinase pathway. Inhibition of both pathways reduces TNF mRNA to control levels. Ethidium bromide stains show equal loading of RNA. These figures are representative of four different experiments.

hibition of either the Erk or p38 kinase pathway only partially reduced cytokine release. Increasing the amounts of each of the inhibitors had no additional effect on cytokine release (data not shown). In contrast, simultaneous inhibition of both pathways reduced cytokine release to near-control levels (Figure 4). These studies strongly suggest that LPS-stimulated AM require activation of both the Erk and the p38 kinase pathways for optimal cytokine release.

LPS-Induced Activation of Both the Erk and the p38 Kinase Pathways Regulates Accumulation of Cytokine mRNAs in Human AM

To determine the effect of the Erk and p38 kinase pathways on accumulation of cytokine mRNAs, we performed Northern blot analysis to detect IL-6 and TNF mRNAs in LPS-stimulated AM. As expected, LPS increased the amounts of IL-6 and TNF mRNAs. Selective inhibition of either the Erk or p38 kinase pathways with PD98059 or SB203580, respectively, partially reduced the accumulation of each of the cytokine mRNAs. The combination of the two inhibitors resulted in a greater reduction in the amounts of the cytokine mRNAs, compared with either inhibitor alone (Figure 5). The observation that inhibition of the p38 kinase pathway reduces cytokine mRNA accumulation contrasts with the data of Lee and associates in monocytes (5). Therefore, we performed similar studies in monocytes. The results of these studies were similar to our studies in AM (Figure 6). These observations suggest that the Erk and p38 kinase pathways regulate either transcription or stability of the cytokine mRNAs.

LPS-Induced Activation of Both the Erk and the p38 Kinase Pathways Is Necessary for Cytokine Gene Transcription in Human AM

To determine whether PD98059 or SB203580 altered transcription of the cytokine genes, we performed nuclear run-on assays to evaluate IL-6 transcription in LPS-stimulated AM. LPS increased levels of IL-6 gene transcription in AM, and inhibition of the Erk kinase pathway with PD98059 or the p38 kinase pathway with SB203580 markedly reduced cytokine gene transcription (Figure 7). These studies suggest that both the Erk and p38 kinase pathways regulate cytokine gene expression, at least in part, at the level of transcription in AM exposed to LPS.

Discussion

One of the major causes of ARDS is sepsis. During sepsis there are increased amounts of LPS in the lungs of affected patients, and LPS is a major stimulus for the release of cytokines during the course of the illness. In these stud-

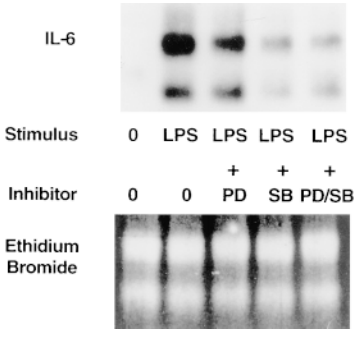

Figure 6. Effect of MAPK inhibition on IL-6 mRNA accumulation in human blood monocytes. LPS increases the accumulation of IL-6 mRNA, and there is only a partial reduction with inhibition of either the Erk or the p38 kinase pathway alone. Inhibition of both pathways simultaneously results in a reduction of mRNA to control levels. Ethidium bromide stains show equal loading of RNA. This figure is representative of three different experiments.

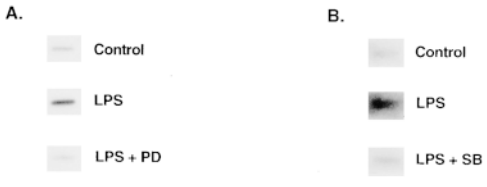

Figure 7. Effect of MAPK inhibition of cytokine gene transcription in human AM. (*A*) LPS increases the transcription of IL-6, and inhibition of the Erk kinases with PD98059 (PD) results in a reduction of transcription to near-control levels. (*B*) LPS increases the transcription of IL-6, and inhibition of the p38 kinases with SB203580 (SB) results in a reduction of transcription to near-control levels. These figures are representative of four different experiments.

ies, we found that both the Erk kinase and p38 kinase pathways are activated in human AM exposed to LPS. Both of these MAPK pathways are activated by LPS alone but this effect is enhanced when AM are cultured in serum, which is known to enhance the effects of LPS. Most importantly, we observed that activation of both the Erk and p38 kinase pathways was necessary for optimal accumulation of IL-6 and TNF mRNAs and cytokine release from LPS-stimulated AM. Inhibition of either of these pathways only partially reduced cytokine gene expression, but simultaneous inhibition of both pathways resulted in a marked reduction in expression of these genes. Nuclear run-on assays showed that one mechanism by which the Erk and p38 kinase pathways regulate accumulation of the cytokine mRNAs is by an effect on transcription. To our knowledge, these are the first studies to show that several MAPK pathways interact positively to regulate expression of cytokine genes. They are also the first studies to show clearly that the Erk kinase pathway regulates cytokine gene expression in response to LPS.

Prior studies by Lee and coworkers (5, 6), using monocytes and LPS to stimulate the cells, showed that inhibition of the p38 kinase pathway with SB203580 resulted in an inhibition of cytokine release. The Erk kinase pathway was not evaluated in these studies. Other stimuli, such as osmotic stress and IL-1, also activate p38 kinases and cytokine gene expression (21). Both osmotic stress and IL-1 increase amounts of cytokine mRNAs in monocytes and THP-1 cells. Inhibition of the p38 kinases with SB203580 results in reduced cytokine release but not a decrease in cytokine mRNA accumulation. Thus, it appears from these studies that SB203580 inhibits the translation of cytokine mRNAs in cells stimulated by a variety of stimuli. The mechanism(s) by which the p38 kinase might regulate translation of cytokine mRNAs is, at present, unknown. It has been postulated that phosphorylation at an AU-rich (AUUUA repeat in the 3′ end of mRNA) site in cytokine mRNAs is important in regulating translation (5). In our studies, we consistently observed a reduction in LPS-induced accumulation of cytokine mRNAs when we inhibited the p38 kinase pathway. This was seen both in AM and in monocytes. By itself, however, inhibition of cytokine mRNA accumulation by SB203580 was only partial. This may explain the differences between our study and prior studies. Our studies clearly show that inhibition of p38 kinases with SB203580 reduces cytokine gene transcription. In this regard, it is known that the p38 kinases also phosphorylate various transcription factors, such as activating transcription factor-2 (ATF2), at sites that increase their transcriptional activity (8, 26, 28). ATF2 is thought to positively regulate, with subunits of nuclear factor (NF)-κB, expression of the promoters of several cytokine genes (29). Another study has also shown that the p38 kinase activates a member of the CAAT/enhancer binding protein (C/EBP) family (30). These observations suggest mechanisms by which the p38 kinases might regulate transcription.

The two important findings in our study are that the Erk kinase pathway is also necessary for optimal LPS-induced cytokine gene expression in human AM, and that there is a positive interaction between the Erk and p38 kinases in regulating LPS-induced cytokine gene expression. Studies have shown that LPS can activate Erk kinases (7, 9). However, to our knowledge, no study has shown that the Erk kinases are involved in LPS-induced cytokine gene expression. The only study that has linked Erk kinases to LPS-induced cytokine gene expression was in BAC-1.2F5 macrophages (15). These cells, which were infected with *v-raf*, released small amounts of cytokines at baseline, and the amount of cytokine mRNAs increased when the cells were exposed to LPS. BAC-1.2F5 macrophages, however, exhibit suppression of LPS-induced Erk kinase activation when infected with *v-raf*. Thus, it is not clear whether the effect of LPS was mediated through the Erk kinases or through some other pathway. Erk kinase activation by IgG or IgE in neutrophils and murine macrophages or mast cells has previously been linked to cytokine (TNF) gene expression (16–18). These studies, however, either did not evaluate Erk kinase interactions with p38 kinases or showed a negative regulation of cytokine gene expression by the p38 kinase (18). Our studies strongly suggest that both Erk and p38 kinases positively interact to regulate cytokine gene expression in human AM.

The mechanism(s) by which Erk kinases regulate LPS-induced cytokine gene expression is unknown. Our study shows that this regulation is, at least in part, at the level of transcription. Various studies have suggested that NF-κB activity can be regulated via phosphorylation (31). It will be of interest in future studies to determine whether LPS induces phosphorylation of subunits of NF-κB, and whether this is regulated by either the Erk or the p38 kinases. Other transcription factors, such as activator protein-1 (AP-1) and C/EBPβ (also known as NF-IL-6) can also mediate activation of the IL-6 and TNF genes (32–40). Many studies have shown that the Erk kinases are necessary for AP-1 activation (32–35) in several types of cells. Erk kinase is thought to increase AP-1 transcriptional activity by induction of *c-fos*, and it phosphorylates c-Jun in N-terminal sites (32). One study has shown that Erk kinases regulate the phosphorylation of NF-IL-6 *in vitro* (41). The Erk kinases are also known to phosphorylate additional transcription factors (42–45), including Elk-1, c-Myc, and cyclic adenosine monophosphate response element binding protein. The role of these transcription factors in mediating cytokine gene expression is less defined.

The JNK kinases are also activated by LPS (10, 11, 13), and they may play a role in regulating cytokine gene expression. This likely is the case because it is known that these kinases regulate the activity of various transcription factors (32, 35, 46–48). We did not pursue the role of the JNK kinases in this study because there are no specific inhibitors for these kinases that can be used in normal cells. Simultaneous inhibition of both the Erk and p38 kinase pathways reduces cytokine gene expression to near-control levels, so our studies suggest that activation of the JNK kinase pathway by itself is not sufficient for expression of the IL-6 and TNF genes.

In summary, human AM require both the Erk and p38 kinase pathways for optimal LPS-induced IL-6 and TNF gene expression. Both the Erk and p38 kinases regulate the expression of cytokine genes, at least in part, at the level of transcription. Thus, these studies suggest that there is a positive interaction between these kinases in LPS-stimulated

AM, unlike the interaction seen in other cells. These studies also suggest that the MAPK pathways play an important role in the inflammatory response that occurs in sepsis-induced acute lung injury.

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